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(54) Title: REAGENT FOR AGGLUTINATION ASSAYS

(57) Abstract

The invention provides a bifunctional recombinant protein comprising a particle-binding antibody or antibody fragment (PBM), and an analyte-binding moiety or molecule (ABM). Preferably, the particle-binding antibody or antibody fragment is an erythrocyte-binding antibody or antibody fragment (EBM), and/or the ABM is selected from the group consisting of an antigenic peptide from an immunodominant region of an env protein of HIV-1 or HIV-2, a gag protein of HIV-1 or HIV-2, and an immunodominant region of the surface antigen of Hepatitis B. Alternatively, the ABM is a single chain Fv region of an antibody directed against an antigen selected from the group consisting of Hepatitis B surface antigen, D-dimer and canine heartworm antigen. In a particularly preferred embodiment, the EBM is a single chain Fv region of an anti-erythrocyte antibody, such as an anti-glycophorin antibody. The protein of the invention is particularly suitable for use in agglutination immunoassays. Methods for producing the protein of the invention, DNA sequences, and assay methods and reagent kits therefor are also provided.

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REAGENT FOR AGGLUTINATION ASSAYS

The present invention relates to a reagent for use in agglutination assays, and in particular whole blood agglutination assays. The invention also relates to a method for detecting an antigen, antibody or other analyte in a sample using the reagent, and to a kit containing the reagent. The invention describes the use of recombinant DNA methods in *E. coli* to produce the key reagents for this assay.

This application claims priority from Australian Provisional Patent Application No. PL 2551, the entire disclosure of which is herein incorporated by reference.

Background of the Invention

Immunoassays and analogous specific binding assays are now very well-known and widely used in a variety of biomedical and other fields. The most commonly used immunoassays utilise complex detection systems involving radioisotopes or enzymes, and suffer from the disadvantage that the assay procedure is lengthy and involved, and requires expensive instrumentation. Radioimmunoassays further suffer from the disadvantage of the radioactive hazard presented by the isotopes. Agglutination immunoassays, using erythrocytes or latex particles as the detection agent, have been proposed as an alternative. Immunoassays and agglutination immunoassays are described in our International Patent Application WO 91/04492, entitled "Agglutination Assay". In particular, our U.S. Patents No. 4,894,347 and No. 5,086,002 describe an agglutination immunoassay designed for use with whole blood samples, in which the endogenous erythrocytes are used as indicating particles, and in which an agglutination reagent comprising an erythrocyte binding molecule conjugated either to an analyte-binding molecule or to an analyte analogue is used.

Non-specific agglutination is avoided if the erythrocyte binding molecule recognises an abundant, well-distributed erythrocyte membrane constituent such as glycophorin. WO 91/04492 describes an autologous agglutination assay of improved sensitivity. The entire disclosures of U.S. Patent No. 4,894,347 and International Patent Application No. WO 91/04492 are also incorporated herein by reference.

Conventional immunoassays, and some agglutination assays, require the isolation of serum or plasma, which in turn usually requires electrical power and specialised equipment, and consequently is very difficult under field conditions or in remote or under-developed areas. It is therefore highly desirable to be able to use a test system which can utilise whole blood, and which requires a minimum of sophisticated apparatus. Test systems for use under field conditions should be stable, rapid, reliable and specific, and should provide a clear-cut demarcation between positive and negative results. In order to be cost effective, such a system should require the minimum number of reagents, which in themselves should be easy to produce. The system described in US-4,894,347 and WO 91/04492, which is marketed as SimpliRED, MicroRED and VetRED tests (Trade Marks of Agen Ltd), meets the requirements of simplicity and ease of use under difficult conditions, and requires a minimum of equipment. It is however, expensive to produce the reagents.

Two main types of reagent are desired for use in these immunoassays, namely antigen-antibody constructs and bispecific antibody constructs. The currently manufactured antigen-antibody reagents utilise, for example, an antigenic peptide from an immunodominant portion of HIV virus (HIV-1 or HIV-2), coupled chemically to the Fab fragment of antibody which is able to bind to glycophorin A on the red cell surface. Alternative reagents of the antigen-antibody type utilise a larger protein, rather than an immunodominant

peptide, for example hepatitis B surface antigen.

Bispecific antibody and Ab-peptide conjugate reagents are currently manufactured by a series of steps involving chemical and enzymic manipulation of antibodies; they consist of two Fab molecules with differing specificities, linked by disulphide bonds at the hinge region. The resultant bispecific $F(ab)_2$ molecule reacts both with an indicator reagent, such as an erythrocyte, and a circulating antigen in a blood sample.

Antibodies (Abs) and Ab fragments can be produced by recombinant DNA technology (Winter and Milstein, Nature, 1991 349 293; U.S. Patent No. 4,946,778 by Ladner *et al*; Australian Patent No. 612,370 by Creative Biomolecules, Inc., using either mammalian cells (Oi, V.T. *et al*, Proc. Natl. Acad. Sci. USA, 1983 80 825-829) or bacteria (Boss, M.A. *et al*, J. Nucl. Acids Res., 1984 12 3791-3806 and also U.S. Patent No. 4,816,397 by Boss *et al*; Cabilly, S., Proc. Natl. Acad. Sci. USA, 1984 81 3273-3277 and European Patent No. 125,023 by Genentech Inc. and City of Hope). In the Fab region the combination of two chains (heavy and light) provides six variable surface loops at the extremity of the molecule. These loops in the outer domain (Fv) are termed complementarity-determining-regions (CDRs), and provide the specificity of binding of the Ab to its antigenic target.

Binding function is localised to the variable domains of the antibody molecule, which are located at the amino terminal end of both the heavy and light chains. The variable regions remain noncovalently associated (as $V_H V_L$ dimers, termed Fv regions) even after proteolytic cleavage from the native antibody molecule, and retain much of their antigen recognition and binding capabilities (see, for example, Inbar *et al*, Proc. Natl. Acad. Sci. USA, 1972 69 2659-2662; Hochman *et al*, Biochem., 1973 12 1130-1135 and Biochem., 1976 15 2706-2710; Sharon and Givol, Biochem., 1976

15 1591-1594; Rosenblatt and Haber, Biochem., 1978 17 3877-3882; Ehrlich *et al*, Biochem., 1980 19 4091-4096). Methods of manufacturing two-chain Fv substantially free of constant region using recombinant DNA techniques are disclosed in US-4,642,334 and corresponding published specification EP-088,994.

We have now found that by using recombinant DNA technology, it is possible to improve and to broaden significantly the applicability of the assay described in US-4,894,347 and WO 91/04492. These references teach the application of either a bispecific antibody F(ab)$_2$ fragment, one half of which binds to erythrocytes and the other to the analyte, or an Fab fragment of the red cell binding antibody attached to a specific peptide. The reagents are manufactured in a series of steps, firstly by digesting the purified mouse antibodies with an enzyme to remove the Fc region, then reduction to Fab, blocking and conjugation. Each stage in the process, but more importantly the entire bioprocess, can be simplified by the use of genetically engineered reagents. For example, oligonucleotide synthesis can provide the gene fragments that encode the various C-terminal peptide tails that constitute analyte specificity. The red cell binding molecule, providing it has sufficient affinity, may be an Fv fragment rather than a complete Fab. Alternatively single chain scFv, or smaller domain structures, can be engineered which may have advantages for product stability and yield. Further improvements to the reagents include the elimination of mouse constant domains, with resulting increased specificity, and improved solubility properties. We have been able to produce bifunctional recombinant proteins comprising an antibody variable region domain, together with either a second antigen-recognising domain or an antigenic region.

Expression systems that are available for the

production of antibody fragments include *E. coli* and alternative prokaryotes, yeast, baculoviral vectors and mammalian cells. We have developed novel *E. coli* secretion vectors which (Power *et al*, Gene 1992 113 95-99) now allow the expression, to exceptionally high levels, of the V_H/V_L/scFv domains of anti-neuraminidase Abs. Downstream processing has been addressed in the context of high-level Ab-domain production. A number of denaturation/ renaturation regimes have been tested, and molecular "flags" incorporated into the expressed antibody domains to aid in purification and conformational assessment. Current physical tests for protein conformation and binding affinity include ELISA, fluorescence quenching, circular dichroism, airfuge centrifugation, and biosensor applications.

We have surprisingly found that the activity of the complementarity determinants at the very ends of IgG Fab arms is maintained, even after 75% of the supporting molecular structure normally present in IgG molecules is removed. The affinity of the antigen-binding domains is not significantly affected; nor does the removal of the supporting molecular structure appear to decrease the stability of the molecule.

The synthesis of antibody variable region domains in recombinant organisms has the potential to enable the production of reagents which might otherwise be impossible to manufacture, such as constructs using large recombinant proteins, many of which are usually produced as insoluble molecules for solid phase assays. Often an antigen will not produce a single immunodominant response in an infected host, and several epitopes from an antigen are necessary to detect circulating antibodies. In such a case, several peptides as a recombinant construct with Ab or fragments avoid the need for the multiple Fab-peptide conjugates. The use of multiple conjugates requires large amounts of blocking reagents to avoid non-specific agglutination resulting from interaction

of the Fab constant regions. Thus, the ability to express the bifunctional molecules in a recombinant host dramatically decreases manufacturing costs for reagents which otherwise would require complex chemical synthesis and additional blocking reagents.

Summary of the Invention

The invention provides an assay, utilizing a series of reagents produced by recombinant DNA technology, that is useful for the detection of drugs, hormones, steroids, antibodies, and other molecules in a biological fluid, particularly in blood. Technology for this assay and these reagents is taught which provides a sensitive assay and a means to produce the key reagents as recombinant antibody molecules, including single chain antibody molecules, in *E. coli*, or in other expression systems known to the person skilled in the art.

According to one aspect of the invention, there is provided a bifunctional recombinant protein comprising a particle-binding antibody or antibody fragment (PBM), and an analyte-binding moiety or molecule (ABM).

The analyte may be an antigen or an antibody. Preferably the particle binding antibody or antibody fragment is an erythrocyte binding antibody or antibody fragment (EBM).

Preferably the ABM is selected from the group consisting of an antigenic peptide from an immunodominant region of the env gp41 protein of HIV-1 or HIV-2, and one of the gag proteins, and an immunodominant region from the surface antigen of Hepatitis B surface antigen. The specific ABMs may be produced by expression from gene fragments, for example, from synthesised oligonucleotides, that encode peptides which constitute the analyte specificity.

In an alternative embodiment, the ABM is a single

chain Fv region of an antibody directed against an antigen selected from the group consisting of Hepatitis B surface antigen, D-dimer and canine heartworm antigen.

Preferably the EBM is a single chain Fv region of an anti-erythrocyte antibody, more preferably an anti-glycophorin antibody.

The use of single chain Fv region in the construct presents the advantage that the constant region of the antibody is almost completely removed, and consequently there is less opportunity for interference by heterophile antibody in the final assay, and the manufacture of a complete reagent is more efficient that would be the case if no blocker antibody were used.

We have found that the orientation of the ABM in relation to the EBM is critical to the sensitivity and specificity of the final product.

In a particularly preferred embodiment, the EBM is the single chain Fv domain of the anti-glycophorin A monoclonal antibody produced by the hybridoma cell line G26.4.1C3/86, which is described in US-4,894,347, and WO91/04492. A sample of this cell line was deposited under the Budapest Treaty at the American Type Culture Collection (12301 Parklawn Drive, Rockville MD, 20852) on 7 September 1988, and received the ATCC accession number HB9893.

In a second aspect of the invention, there is provided a DNA sequence encoding as a single transcriptional unit a particle-binding moiety operatively linked to an analyte-binding moiety, as well as expression vectors and host cells comprising said sequence.

A third aspect of the invention provides assay methods and kits utilising the recombinant protein of the invention.

Although the use of an Fv region of an antibody is preferred, it should be clearly understood that the invention

includes within its scope the use of Fav, $F(ab)_2$ or V_H fragments of antibodies.

The host cell may be any of those currently used by those skilled in the art of expression in recombinant organisms, and is preferably *E. coli*. However, it will be clearly understood that other hosts, such as other bacteria, yeasts or insect, mammalian or plant cells may be used. The *E. coli* expression vectors described herein are novel, particularly with respect to the design of protease-resistant 'tails' with the unique features required by the diagnostic test. We have optimised the induction regime and fermentation conditions for high-yielding production.

The DNA encoding both the erythrocyte binding activity and the specific analyte binding activity may be located on a DNA element capable of replication and the expression of the genes for the bifunctional reagents. This DNA element may be a plasmid or any equivalent DNA element capable of replication and expression in an appropriate host.

The portion of the bifunctional reagent which has specific analyte binding activity may be encoded by DNA which has been produced from cells and tissues by any of the standard techniques known in the art for the amplification of DNA, such as the polymerase chain reaction, the ligase chain reaction, or isothermal amplification.

The use of recombinant bifunctional reagents provides the following advantages:

1. Simplification of current production procedures: No chemical coupling through disulphide bonds is necessary.

The bifunctional fusion protein is made as a single polypeptide chain.

2. Any two of a wide range of analyte-binding molecules can be incorporated into a bifunctional single

polypeptide chain.

3. Ease of manipulation to produce modified bifunctional single polypeptide chain by mutation of the DNA.

4. No batch to batch variation.

5. Expression from host cells produced large amounts of soluble polypeptide.

6. Ease of identification, isolation and purification.

Less expensive to produce.

7. Increased scope of bifunctional reagents.

8. No dependence on high levels of protein production from hybridomas.

9. Recombinant DNA techniques make infinite permutations possible.

Detailed Description of the Invention

In the agglutination assay of this invention, a recombinant reagent is provided which is derived from cloned DNA coding for the erythrocyte binding antibody, which as a result of genetic manipulation is fused to an analyte binding molecule encoded by the gene or gene fragment for the specific analyte binding molecule without substantially changing the binding characteristics of the binding portion. The reagent is non-agglutinating when incubated with endogenous erythrocytes in the absence of the analyte.

The invention will be described in detail by way of reference only to the following non-limiting examples, and to the drawings in which:

Figure 1 illustrates the sequence of the IgG (1C3/86) gamma chain derived from clone gamma1.1.1a. The nucleotide and deduced amino acid sequence (mature sequence shown in bold type and single letter code) of 1C3/86 IgG gamma-1.1.1a are shown;

Figure 2 illustrates the sequence of the IgG

(1C3/86) kappa chain derived by PCR amplification and clone K4AC1/C2. The nucleotide and deduced amino acid sequence (shown in bold type and single letter code) of mature 1C3/86 IgG kappa chain (sequence is a composite of that determined from clones K4AC1/C2 and the gene amplified directly from mRNA by polymerase chain reaction) are shown;

Figure 3 illustrates the strategy for the amplification of 1C3/86 gamma and kappa gene variable domains and the construction of the scFv in expression vector pPOW. PCR primer-template combinations used to amplify various antibody fragments are shown.

Figure 4 illustrates the strategy for the amplification and cloning of 1C3/86 scFv in expression vector pHFA. PCR primer-template combinations used to amplify various antibody fragments are shown.

Figure 5 illustrates the strategies for amplification and cloning of scFv's with combined FLAG and HIV immunodominant peptide epitopes in the expression vector $pHFA_{sac}$. PCR primer-template combinations used to amplify various antibody fragments are shown.

Figure 6 illustrates the vectors pPOW, pHFA and $pHFA_{sac}$ used for the construction and expression of the 1C3/86 scFv (described in figures 3,4 and 5) with pertinent cloning sites. Amp^r; ampicillin resistance gene, ColE1 or Ori; *E.coli* origin of replication M13 ORI; M13 phage origin of replication, Gene3; gene 3 phage surface protein gene, Amber; amber stop codon,(TAG) fD; transcription terminator, placZ; lacZ promoter, *cI*857; lambda heat labile repressor gene, P_r and P_l; lambda phage right and left promoters, FLAG; gene for epitope recognised by M2 anti-flag IgG and pelB; gene for pectate lyase signal sequence.

Figure 7 illustrates the protein sequences of peptide epitopes which may be generated by PCR reaction and linked in the reaction or added by recombinant DNA

techniques.

Figure 8 illustrates the activity of the recombinant protein in ELISA assays.

The anti-glycophorin A monoclonal antibody 1C3/86 was selected as a model antibody. The gene encoding 1C3/86 IgG was cloned into an *Escherichia coli* host, and the nucleotide sequence of the antibody was determined. Synthetic oligonucleotide primers were designed in order to enable the variable domains of the antibody to be cloned, linked together to form a single chain Fv domain (scFv), into various expression vectors. Various peptide epitopes were added to the C-terminus of the scFv molecule.

Example 1

Isolation and Characterisation of Genes Encoding Antibody Fragments

A strategy utilising the polymerase chain reaction (PCR) to identify segments of the genes encoding the antibody and to add linkers and peptide epitopes to those segments to form single chain, antibody-based reagents was adopted.

a) Messenger RNA (mRNA) was prepared from a monoclonal cell line (1C3/86), referred to above, which produced anti-erythrocyte IgGs which bound with high affinity to RBCs but did not produce auto-agglutination.

From this mRNA template, single and double stranded complementary DNA (ss- and ds-cDNA respectively) were synthesised. The ds-cDNA was cloned into lambda-gt10 arms and packaged into a phage library. The heavy chain clone gamma-M/1.1 (Tyler et al, Proc. Natl. Acad. Sci., 1982 79 2008-2012) and the light chain clone pH76-kappa-10 (Adams et al, Biochem., 1980 19 2711-2719) were used to source ds-DNA inserts for the screening of the gt10 library. Positive clones were amplified, and the positive insert cDNA sub-

cloned into pUC18. As a result, a near full-length gamma clone (gamma-1.1.1a) was identified, the nucleotide sequence was determined and from this the protein sequence was deduced (Figure 1). The sequences of a partial kappa clone (kappa-4AC1) which encoded the 3' end of the variable domain and full constant domain were determined in a similar fashion.

To determine the nucleotide sequence of the 1C3/86 kappa light chain at the 5' end, the following approach was adopted. A mixed N-terminal sequence (see below) was first determined for the intact 1C3/86 Ig in an Applied Biosystems sequencer.

mixed sequence D/E I/V V/R M/L S/L Q/E S/S P/G S/G (automatic sequencer)

From the mixed amino acid sequence above and the sequence deduced from a gamma heavy chain clone as follows:

gamma chain E V R L L E S G G (clone 1.1.1a)

the N-terminus of the variable region of the kappa light chain, not present in gt10 library clones, was determined to be;

kappa chain D I V M S Q S P S (deduced by difference)

From this sequence for the N-terminus of the kappa chain above an approximation of the 5' light chain variable region was compiled by applying common usage triplet codes found in IgG genes (see oligonucleotide N960 in Table 1 below). The light chain variable region gene was then amplified by PCR using the redundant, forward (sense) primer

N960 and the reverse (antisense) primer N852 (see Table 1), which was based on the kappa constant region beginning at nucleotide 337 (see Figure 2), as described by Chiang *et al*, Biotechniques, 1989 7 360-366. The amplification reaction yielded a single product, which when cloned and sequenced showed a coding sequence consistent with a kappa light chain and identical at the 3' end with the overlapping kappa clone K4AC1. The sequences derived from PCR and gt10 library enabled the compilation of the sequence shown in Figure 2.

Table 1

Forward (sense) oligonucleotides:

N 907	GGG GTC GCG GAG GTG AGG CTT CTC
N 960	CCC GCC AGA CGT/C GAT/C ATT/C GTG/C ATG
N 978	CCC ACG GTC ACC GTC GCC TCC GGT GGT GGT GGT TCA GGA GGA GGA GGT
N 979	TCA GGA GGA GGA GGT TCG GGT GGT GGT GGT TCG GAC ATC GTC ATG
N1237	AA AAA GCG GCC CAG CCG GCC ATG GCC GAG GTG AGG CTT CTC GAG
N1479	TCT GGA GGT GGC CCG GTA CAA CCT GGA GGA TCT CTG AAA CTC TCC
N1617	ATG GCG GAG GTG AGG CTT CTT GAG TCT GGA GGT GGC CCG G
N*Sfi15*	CAT GCC ATG ACT CGC GGC CCA GCC GGC CAT GGC C(C/G)A GGT (C/G)(A/C)A (A/G)CT GCA G(C/G)A GTC (A/T)GG

Reverse complementary oligonucleotides:

N 852	CC GAA TTC GAT GGA TAC AGT TGG TGC AGC ATC AGC CCG
N 908	GAC GGC CAG GAT ACG GCC GGC GGA GAC GGT GAC CAG AGT
N 909	GCA GCC CCA GAT GCC CAG CAG CTG CTG ATC TTT CAG ATA ACG TTC GAC GGC CAG GAT ACG
N 911	GAC GGC CAG GAT ACG CCG TTT AAT CTC GAG CTT GGT GCC
N 976	GGG GAA TTC TTA AGA CGC ATT CCA CGG GAC CGC CGT GGT GCA GAT
N1294	GAC GGC CAG GAT ACG TTT ATC ATC ATC ATC
N1296	GAC CGC CGT GGT GCA GAT CAG TTT GCC AGA GCA GCC CCA GAT GCC
N1645	AAA AAA CCG CGG GAA TTC TTA AGA CGC ATT CC
N1646	AAA AAA CCG CGG GAA TTC TTA ACA CAC CTG TC

NVKFORNOT[b] equimolar mixture of:

GAG TCA TTC TGC GGC CGC CCG TTT GAT TTC CAG CTT GGT GCC
GAG TCA TTC TGC GGC CGC CCG TTT TAT TTC CAG CTT GGT CCC
GAG TCA TTC TGC GGC CGC CCG TTT TAT TTC CAA CTT TGT CCC
GAG TCA TTC TGC GGC CGC CCG TTT CAG CTC CAG CTT GGT CCC

Primers are 5' to 3' (left to right). Forward (sense) oligonucleotides translate to the amino acid sequence of the expressed protein segment whereas reverse primers need to be reversed and complemented.

[a,b]"Making antibodies in bacteria and on phage", EMBO Practical Course Manual, IRBM, Pomezia, Italy

b) A single chain antibody fragment (scFv) was constructed from the 1C3/86 molecule as follows:

i) Amplification and cloning of the heavy-chain variable domain.

1. The amplification of genes and synthesis of DNA sequences in these genes for cloning were performed by application of the polymerase chain reaction (PCR) as follows. A typical reaction (100 l volume) contained 1-10 ng of template DNA, 1-2 U of thermostable DNA polymerase, 5 l of a mixed A,C,G and T deoxynucleotide (dNTP solution) with each base at a concentration of 2 mM, 5 l of each terminal primer (10 pMolar each) and, where used, 1 l of internal primers (0.05-0.1 pM), Mg++ to a final concentration of 1-5 mM, a reaction buffer appropriate for the particular polymerase chosen (supplied by manufacturer), and water to 100 l. The reactants were mixed and overlayed with paraffin oil (Sigma biochemicals) and subjected to 25-30 cycles in a thermal cycler (Corbett Research, Australia). The general strategy for each of the examples in Figures 3, 4 and 5 consisted of a denaturation step at 93°C (usually 1 minute), an annealing step between 50 and 65°C for 1 minute and an extension step at 72°C for 2 minutes. Annealing temperatures were adjusted as required to give final product.

2. Oligonucleotide primers (Table 1) were synthesized to amplify the variable domain (V_h) from the

heavy chain cDNA clone gamma-1.1.1a, , and to add a *Tha*1 restriction site at the 5' end (N907) and a *Bst* E2 - peptide epitope -*Eco* R1 sequence at the 3' end (N908/N909/N1296/N976), as described in Figure 3A. The product was digested with *Tha* 1 and *Eco* R1, and cloned into the *Msc* 1/*Eco* R1-digested expression vector pPOW (Power *et al*, Gene., 1992 113 95-99) (Figure 6A), and transformed into *E coli* strain TG-1 (Gibson T.J., 1984, "Studies on the Epstein-Barr virus genome", Ph.D. thesis, Cambridge University, England) .

3. Transformed *E coli* were screened for the presence of plasmids carrying the V_h gene fragment and selected clones (hereafter referred to as pPOW1C3Vh$_{HIV1}$) were sequenced to check the integrity of the cloning procedure. These clones are identified in PL 2551 as pP1C3Vh

ii) Amplification and cloning of the light chain variable domain and construction of composite single-chain antibody (scFv) reagents

1. Oligonucleotide primers were synthesized to simultaneously amplify (as in i(1) above) and add to the cloned light chain gene, in a PCR amplification reaction, a *Bst* E2 site and a sequence coding for a linker (amino acid sequence -$(GGGGS)_3$-) at the 5' end (N978/N979) and a peptide epitope-*Eco* R1 sequence at the 3' end (N911/N909/N1296/N976) as described in Figure 3B.

2. The V_l product described in Figure 3B was digested with *Bst* E2 and *Eco* R1 and cloned into the Bst E2/Eco R1 digested plasmid construct, pPOW1C3scVh$_{HIV1}$ above (Figure 3A).

3. Transformed *E. coli* (TG1) were screened for the presence of the V_h and V_l sequences and selected clones, hereafter referred to as pPOW1C3scFv$_{HIV1}$(Figure 3C), were partly sequenced to check the integrity of the cloning

procedure. These clones are identified in PL 2551 as pP1C3scFv.

4. Oligonucleotide N*Sfi15* and *NVKFORNOT* (Table 1) were used to add *Sfi* 1 and *Not* 1 restriction sites (by PCR amplification) to the 5' and 3' ends respectively of the scFv gene construct in pPOW1C3scFv$_{HIV1}$(see Figure 4) - in this amplification, the gp41 HIV1 epitope was removed. The PCR product was digested with these restriction enzymes and cloned into the likewise restricted vector pHFA (see Figure 6B) which contains the alternative octapeptide FLAG tag (Figure 7A) - pHFA is the parent of the vector pHEN (Hoogenboom *et al*, 1991) . The construct was then transferred into the *E. coli* strain HB2151, a strain in which the nucleotide sequence TAG (amber mutation) is recognised as a stop codon . Clones, referred to as pHFA1C3scFv$_{FLAG}$, were identified by hybridization, were sequenced, and were tested for expression of a scFv-peptide fusion as evidenced by reactivity to the M2-anti FLAG antibody (IBI Corp., USA).

5. The HIV1 and HIV2 epitopes (Figures 7B and 7C) -were added back to the scFv to give plasmid constructs pHFA$_{sac}$1C3scFv$_{FLAG/HIV1}$ and pHFA$_{sac}$1C3scFv$_{FLAG/HIV2}$ (Figures 5A and 5B respectively). In this procedure, the FLAG epitope and alternative HIV epitopes were added to the scFv gene in pHFA described in ii)4 above by PCR amplification. The sequence changes to the Vh gene, introduced in pHFA constructs, were returned to the native and a *Bam*H1 restriction site adjacent to the 5' end of the gene was removed by the use of the forward oligonucleotides N1479, N1617 and N1237. At the 3'end of the FLAG sequence terminating the scFv gene construction in pHFA, oligonucleotides N1294, N909, N1296, N976 and N1645 introduced a HIV1 epitope, *Eco* R1 and *Sac* 2 sites. In a similar fashion the HIV2 epitope and restriction sites were added with 3' oligonucleotides N1297, N1311, N1310 and N1646 (Table 1). PCR products were

restricted with *Sfi* 1 and *Sac* 2 were cloned into the likewise restricted vector $pHFA_{SAC}$ (Figure 6C), a derivative of pHFA. In this procedure, the FLAG sequence in the vector was deleted and replaced with the FLAG sequence of the PCR construct but was between the scFv and the HIV epitopes - a TAA stop codon was included so that in suppressor or non-suppressor cell lines, translation would terminate after the HIV epitope.

c) Expression of recombinant scFv

Recombinant *E. coli* were grown in 10mls of 2X-YT medium (10 gm yeast extract, 15 gm tryptone, 5 gm NaCl per litre) overnight at 30°C in the case of pPOW constructs, and at 37° in the case of pHFA constructs. Overnight cultures were diluted to an OD_{600} of 0.05 into 100ml of fresh medium (0.1% glucose was included in the case of pHFA constructs) and grown to mid-log phase (OD_{600} 0.5-0.9).

Cultures of pHFA plasmids were induced upon the addition of isopropyl- -D-thiogalactopyranoside; (IPTG; Sigma 15502) to a concentration of 1mM, and growth continued at 30°C as required.

Cultures of pPOW were induced by raising the temperature of the medium to 42°C for 15 minutes, after which the incubation was continued at 37°C for 2-4 hours.

Levels of recombinant proteins in the *E. coli* periplasmic space and the culture supernatant in each case were assayed by ELISA, Western blots of SDS-PAGE gels, and by the agglutination assay described below.

Example 2

Activity and Expression Levels of Recombinant scFv

i) Western analysis

Periplasmic proteins were isolated by suspending the *E. coli* in 25% w/v sucrose/10 mM Tris-HCl (pH 7.5) and 16 mM EDTA. Cells were then collected by centrifugation and

resuspended in ice-cold water. The particulate material and the soluble fractions were analysed by SDS-PAGE followed by Western blot. Active expression was assessed by the presence of a product of apparent M_r 30 Kd. Mouse antibodies directed against the C-terminal FLAG peptide (M2 anti FLAG) or HIV epitopes (1B1 or 2B4), included in scFv constructs, were used as primary antibodies in this analysis, and were detected with horse-radish peroxidase bound to goat anti-mouse IgG in the normal manner.

ii) ELISA assay

Supernatants from *E. coli* cultures and purified scFv reagents were assayed by ELISA. The assay was performed as follows:

1. "Nuclon" plates were coated overnight with 100 l of 10 g/ml human glycophorin-A (Sigma) in PBS.
2. Washed plate wells 3X with PBS.
3. Block with 200 l of 2% (W/V) skim milk in PBS for 2h.
4. Wash 3X with PBS.
5. Add 20 l 10% (W/V) skim milk in PBS and 80 l culture fluid or purified antibody in PBS and incubate at 20°C for 20 mins.
6. Wash 3X with PBS/0.05% (V/V) Tween-20
7. Wash 3X with PBS
8. Add 100 l of 2 g/ml anti-tag antibody in PBS/2%(W/V) skim milk powder and incubate for 60 mins at 20°C
9. Repeat steps 6 and 7
10. Add 100 l of 1-2 g/ml HRP-goat anti-mouse IgG antibody in PBS/2%(W/V) skim milk powder and incubate for 60 mins at 20°C
11. Wash as in steps 6 and 7
12. Add 100 l of activated ABTS solution (see below) and develop for 30 mins at 20°C

13. Quench by adding 50 1 of of 3.2 g/l of sodium fluoride and read at 405nm

ABTS (2,2 azino di(3-ethyl)benzthiazoline sulfonic acid) solution (25 ng/ml): 0.25g ABTS

10 ml H_2O

Store in a dark bottle at 4°C and dilute 1:50 in citrate buffer for use.

Citrate buffer 0.1M pH 4: 2.58g citric acid

2.18g Na_2HPO_4

make up to 200ml with distilled water and adjust pH to 4.

Activated ABTS: add 10 1 of 30% hydrogen peroxide to 10ml of diluted ABTS solution.

iii) Agglutination assay

Aliquots of culture isolates or supernatants (10-500 1) which contained the scFv were mixed with 10 1 of whole blood (for volumes greater than 100 1, the mixture was gently mixed for 15 min and the sensitised cells collected by centrifugation and re suspended in PBS). Simultaneously, 20 1 of a second antibody (25 g/ml) directed against the C-terminal epitope of the scFv was added and the mixture stirred briefly with a plastic rod. The level of the recombinant scFv was assessed against negative and positive controls by the rate and degree of agglutination over a two minute period.

Example 3

Functional Epitopes linked to scFv Antibody

Epitopes of the surface protein gp41 from HIV1 and HIV2 virus types may be combined with epitopes from gp120 surface protein or p24 core protein or substituted for the M2-FLAG epitope in scFv constructs or added to the scFv-M2

FLAG construct, thereby producing various bifunctional reagents capable of binding erythrocytes and serum antibodies which may be present in patient's serum. The sequences of the M2-FLAG, HIV1 and HIV2 epitopes are shown in Figure 7.

Example 4

Expression of Active scFv Antibody

When cultured under the conditions described in Example 1, the host cells expressed scFv antibody protein, which was transported through the host cell membranes to the periplasmic space and culture supernatant.

Example 5

Activity and Specificity of the Recombinant scFv Antibody

The recombinant 1C3/86 scFv-FLAG efficiently agglutinates erythrocytes in an assay which uses monoclonal antibody directed against the M2-FLAG epitope (IBI Corp, U.S.A.) as the cross-linking moiety, with activity comparable to that shown by the prior art SimpliRED assay, in which a synthetically produced conjugate of the HIV-1 gp41 immunodominant epitope and 1C3/86 Fab was used as the reagent, and antibody 1B1/114 was used as a known positive sample. Constructs with either HIV-1 or HIV-2 sequences were effective also in mediation of agglutination when respective monoclonal antibodies 1B1 or 2A6 and 2B4 (for HIV1 and HIV2 respectively) were included in the assay.

Example 6

Affinity of the Recombinant Antibody

The scFv antibody recognises glycophorin A with comparable affinity to Fab, as judged by ELISA assay. These results are illustrated in Figure 8.

Example 7

Anti-human red cell, single chain Fv fragment linked to HIV-1 peptide with "flag" peptide (scFvflagHIV-1) was tested with 29 HIV-1 confirmed seropositive samples and 22 seronegatives. All of these samples were correctly identified in agglutination tests, and the results were in agreement with those obtained using a chemically-constructed Fab-peptide conjugate. Similarly, scFvflagHIV-2 was tested with 18 confirmed seropositive samples; 22 seronegtives and all samples were correctly detected, which was also in agreement with results obtained with a chemical construct. The results are summarised in Table 2.

Table 2

REAGENT	SENSITIVITY	SPECIFICITY
	HIV-1 Seropositive	HIV-1/2 Seronegative
scFvflagHIV-1	100% (29/29)	100% (22/22)
Chemical HIV-1	100% (29/29)	100% (22/22)
	HIV-2 Seropositive	HIV-1/2 Seronegative
scFvflagHIV-2	100% (18/18)	100% (22/22)
Chemical HIV-2	100% (18/18)	100% (22/22)

Example 8

A Hepatitis B surface antigen binding fragment may be substituted for the HIV-binding peptide, thereby producing a bifunctional reagent which has the capacity to bind a different analyte, in this case Hepatitis B and in so doing to agglutinate the erythrocytes.

References cited herein are listed on the following pages.

It will be clearly understood that the invention in its general aspects is not limited to the specific details referred to hereinabove.

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CLAIMS:

1. A bifunctional recombinant protein comprising a particle-binding antibody or antibody fragment (PBM), and an analyte-binding moiety or molecule (ABM).

2. A bifunctional recombinant protein according to claim 1 in which the particle-binding antibody or antibody fragment is an erythrocyte-binding antibody or antibody fragment (EBM).

3. A bifunctional recombinant protein according to claim 1 or claim 2 in which the ABM is selected from the group consisting of an antigenic peptide from an immunodominant region of an env protein of HIV-1 or HIV-2, a gag protein of HIV-1 or HIV-2, and an immunodominant region of the surface antigen of Hepatitis B.

4. A bifunctional recombinant protein according to claim 1 or claim 2 in which the ABM is a single chain Fv region of an antibody directed against an antigen selected from tne group consisting of Hepatitis B surface antigen, D-dimer and canine heartworm antigen.

5. A bifunctional recombinant protein according to any one of the preceding claims in which the EBM is a single chain Fv region of an anti-erythrocyte antibody.

6. A bifunctional recombinant protein according to claim 5 wherein the anti-erythrocyte antibody is an anti-glycophorin antibody.

7. A bifunctional recombinant protein according to claim 6 wherein the EBM is the single chain Fv domain of the anti-glycophorin A monoclonal antibody produced by the hybridoma G26.4.1C3/86 (ATCC number HB9893).

8. A DNA sequence encoding as a single transcriptional unit a particle-binding antibody or antibody fragment (PBM) operatively linked to an analyte-binding moiety or molecule (ABM).

9. An expression vector comprising a DNA sequence according to claim 8.

10. A host cell comprising a DNA sequence according to claim 8.

11. A host cell according to claim 8 which is *Escherichia coli*.

12. A DNA element capable of replication and expression, comprising a DNA sequence according to claim 8.

13. A DNA element according to claim 12 which is a plasmid.

14. A specific binding assay for detection of an analyte, comprising as a detection agent a bifunctional recombinant protein according to any one of claims 1 to 7.

15. A specific binding assay according to claim 14 which is an immunoassay.

16. A specific binding assay according to claim 14 which is an agglutination immunoassay.

17. A kit of reagents adapted for use in a specific binding assay according to any one of claims 14 to 16.

18. A method of preparing a bifunctional recombinant protein according to any one of claims 1 to 7 comprising the step of utilising a DNA sequence according to claim 8.

19. A method of preparing a bifunctional recombinant protein according to claim 1, comprising the steps of:

a) preparing a DNA sequence encoding a complementarity determining region of an antibody specific for a particle;

b) Preparing a DNA sequence encoding an analyte-binding protein;

c) operatively linking the DNA sequences from step a) and step b) under the control of transcriptional and translational regulators;

d) transferring the product of step c) into a host organism;

e) permitting the host organism to express the DNA sequences; and

f) recovering the protein.

20. A method according to claim 19 in which the antibody specific for a particle is an anti-erythrocyte antibody.

21. A method according to claim 20 in which the anti-erythrocyte antibody is an anti-glycophorin antibody.

22. A method according to any one of claims 19 to 21 in which the analyte-binding protein is selected from the group consisting of an antigenic peptide from an immunodominant region of an env protein of HIV-1 or HIV-2, a gag protein of HIV-1 or HIV-2, and an immunodominant region of the surface antigen of Hepatitis B.

23. A method according to any one of claims 19 to 21 in which the analyte-binding protein is an antibody specific for an antigen which is selected from the group consisting of Hepatitis B surface antigen, D-dimer, and canine heartworm antigen.

Figure 1.

```
     M   D   F   G   L   I   F   F   I   V   A   L   L   K
1    ATG GAT TTT GGG CTG ATT TTT TTT ATT GTT GCT CTT TTA AAA

     G   V   Q   C   E   V   R   L   L   E   S   G   G   G
43   GGG GTC CAG TGT GAG GTG AGG CTT CTC GAG TCT GGA GGT GGC

     P   V   Q   P   G   G   S   L   K   L   S   C   A   A
85   CCG GTA CAA CCT GGA GGA TCC CTG AAA CTC TCC TGT GCA GCC

     S   G   F   D   F   S   R   Y   W   M   N   W   V   R
127  TCA GGA TTC GAT TTT AGT AGA TAC TGG ATG AAT TGG gtc CGG

     R   A   P   G   K   G   L   E   W   I   G   E   I   N
169  CGG GCT CCA GGG AAG GGG CTA GAG TGG ATT GGA GAA ATT AAT

     Q   Q   S   S   T   I   N   Y   S   P   P   L   K   D
211  CAA CAA AGC AGT ACG ATA AAC TAT TCG CCA CCT CTG AAG GAT

     K   F   I   I   S   R   D   N   A   K   S   T   L   Y
253  AAA TTC ATC ATC TCC AGA GAC AAC GCC AAA AGT ACG CTG TAC

     L   Q   M   N   K   V   R   S   E   D   T   A   L   Y
295  CTG CAA ATG AAC AAA GTG AGA TCT GAG GAC ACA GCC CTT TAT

     Y   C   A   R   L   S   L   T   A   A   G   F   A   Y
337  TAT TGT GCA AGA CTT TCT CTT ACT GCG GCA GGG TTT GCT TAC

     W   G   Q   G   T   L   V   T   V   S   A   A   K   T
379  TGG GGC CAA GGG ACT CTG GTC ACT GTC TCT GCA GCC AAA ACG

     T   P   P   S   V   Y   P   L   A   P   G   S   A   A
421  ACA CCC CCA TCT GTC TAT CCA CTG GCC CCT GGA TCT GCT GCC

     Q   T   N   S   M   V   T   L   G   C   L   V   K   G
463  CAA ACT AAC TCC ATG GTG ACC CTG GGA TGC CTG GTC AAG GGC

     Y   F   P   E   P   V   T   V   T   W   N   S   G   S
505  TAT TTC CCT GAG CCA GTG ACA GTG ACC TGG AAC TCT GGA TCC

     L   S   S   G   V   H   T   F   P   A   V   L   Q   S
547  CTG TCC AGC GGT GTG CAC ACC TTC CCA GCT GTC CTG CAG TCT

     D   L   Y   T   L   S   S   S   V   T   V   P   S   S
589  GAC CTC TAC ACT CTG AGC AGC TCA GTG ACT GTC CCC TCC AGC

     T   W   P   S   E   T   V   T   C   N   V   A   H   P
631  ACC TGG CCC AGC GAG ACC GTC ACC TGC AAC GTT GCC CAC CCG

     A   S   S   T   K   V   D   K   K   I   V   P   R   D
673  GCC AGC AGC ACC AAG GTG GAC AAG AAA ATT GTG CCC AGG GAT

     C   G   C   K   P   C   I   C   T   V   P   E   V   S
715  TGT GGT TGT AAG CCT TGC ATA TGT ACA GTC CCA GAA GTA TCA
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Figure 1 continued

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      S   V   F   I   F   P   P   K   P   K   D   V   L   T
757   TCT GTC TTC ATC TTC CCC CCA AAG CCC AAG GAT GTG CTC ACC

      I   T   L   T   P   K   V   T   C   V   V   V   D   I
799   ATT ACT CTG ACT CCT AAG GTC ACG TGT GTT GTG GTA GAC ATC

      S   K   D   D   P   E   V   Q   F   S   W   F   V   D
841   AGC AAG GAT GAT CCC GAG GTC CAG TTC AGC TGG TTT GTA GAT

      D   V   E   V   H   T   A   Q   T   Q   P   R   E   E
883   GAT GTG GAG GTG CAC ACA GCT CAG ACG CAA CCC CGG GAG GAG

      Q   F   N   S   T   F   R   S   V   S   E   L   P   I
925   CAG TTC AAC AGC ACT TTC CGC TCA GTC AGT GAA CTT CCC ATC

      M   H   Q   D   W   L   N   G   K   E   F   K   C   R
967   ATG CAC CAG GAC TGG CTC AAT GGC AAG GAG TTC AAA TGC AGG

      V   N   S   A   A   F   P   A   P   I   E   K   T   I
1009  GTA AAC AGT GCA GCT TTC CCT GCC CCC ATC GAG AAA ACC ATC

      S   K   T   K   G   R   P   K   A   P   Q   V   Y   T
1051  TCC AAA ACC AAA GGC AGA CCG AAG GCT CCA CAG GTG TAC ACC

      I   P   P   P   K   E   Q   M   A   K   D   K   V   S
1093  ATT CCA CCT CCC AAG GAG CAG ATG GCC AAG GAT AAA GTC AGT

      L   T   C   M   I   T   D   F   F   P   E   D   I   T
1135  CTG ACC TGC ATG ATA ACA GAC TTC TTC CCT GAA GAC ATT ACT

      V   E   W   Q   W   N   G   Q   P   A   E   N   Y   K
1177  GTG GAG TGG CAG TGG AAT GGG CAG CCA GCG GAG AAC TAC AAG

      N   T   Q   P   I   M   D   T   D   G   S   Y   F   V
1219  AAC ACT CAG CCC ATC ATG GAC ACA GAT GGC TCT TAC TTC GTC

      Y   S   K   L   N   V   Q   K   S   N   W   E   A   G
1261  TAC AGC AAG CTC AAT GTG CAG AAG AGC AAC TGG GAG GCA GGA

      N   T   F   T   C   S   V   L   H   E   G   L   H   N
1303  AAT ACT TTC ACC TGC TCT GTG TTA CAT GAG GGC CTG CAC AAC

      H   H   T   E   K   S   L   S   H   S   P   G   K   *
1345  CAC CAT ACT GAG AAG AGC CTC TCC CAC TCT CCT GGT AAA TGA
```

Figure 2.

```
     D   I   V   M   S   Q   S   P   S   S   L   A   V   S
1    GAC ATC GTC ATG TCA CAG TCT CCA TCC TCC CTG GCT GTG TCA

     V   G   E   K   V   S   M   S   C   K   S   S   Q   S
43   GTA GGA GAG AAG GTC AGT ATG AGC TGC AAA TCC AGT CAG AGT

     L   F   N   S   R   T   R   K   N   Y   L   T   W   Y
85   CTG TTC AAC AGT AGA ACC CGA AAG AAC TAC TTG ACT TGG TAC

     Q   Q   K   P   G   Q   S   P   K   P   L   I   Y   W
127  CAG CAG AAA CCA GGG CAG TCT CCT AAA CCG CTG ATC TAC TGG

     A   S   T   R   E   S   G   V   P   D   R   F   T   G
169  GCA TCC ACT AGG GAA TCT GGG GTC CCT GAT CGC TTC ACA GGC

     S   G   S   G   T   D   F   T   L   T   I   S   S   V
211  AGT GGA TCT GGG ACA GAT TTC ACT CTC ACC ATC AGC AGT GTG

     Q   A   E   D   L   A   D   Y   Y   C   K   Q   S   Y
253  CAG GCT GAA GAC CTG GCA GAT TAT TAC TGC AAG CAA TCT TAT

     N   L   R   T   F   G   G   G   T   K   L   E   I   K
295  AAT CTT CGG ACG TTC GGT GGA GGC ACC AAG CTG GAA ATT AAA

     R   A   D   A   A   P   T   V   S   I   F   P   P   S
337  CGG GCT GAT GCT GCA CCA ACT GTA TCC ATC TTc CCA CCA TCC

     S   E   Q   L   T   S   G   G   A   S   V   V   C   F
379  AGT GAG CAG TTA ACA TCT GGA GGT GCC TCA GTC GTG TGC TTC

     L   N   N   F   Y   P   K   D   I   N   V   K   W   K
421  TTG AAC AAC TTC TAC CCC AAA GAC ATC AAT GTC AAG TGG AAG

     I   D   G   S   E   R   Q   N   G   V   L   N   S   W
463  ATT GAT GGC AGT GAA CGA CAA AAT GGC GTC CTG AAC AGT TGG

     T   D   Q   D   S   K   D   S   T   Y   S   M   S   S
505  ACT GAT CAG GAC AGC AAA GAC AGC ACC TAC AGC ATG AGC AGC

     T   L   T   L   T   K   D   E   Y   E   R   H   N   S
547  ACC CTC ACG TTG ACC AAG GAC GAG TAT GAA CGA CAT AAC AGC

     Y   T   C   E   A   T   H   K   T   S   T   S   P   I
589  TAT ACC TGT GAG GCC ACT CAC AAG ACA TCA ACT TCA CCC ATT

     V   K   S   F   N   R   N   E   C   *
631  GTC AAG AGC TTC AAC AGG AAT GAG TGT TAG
```

Figure 3

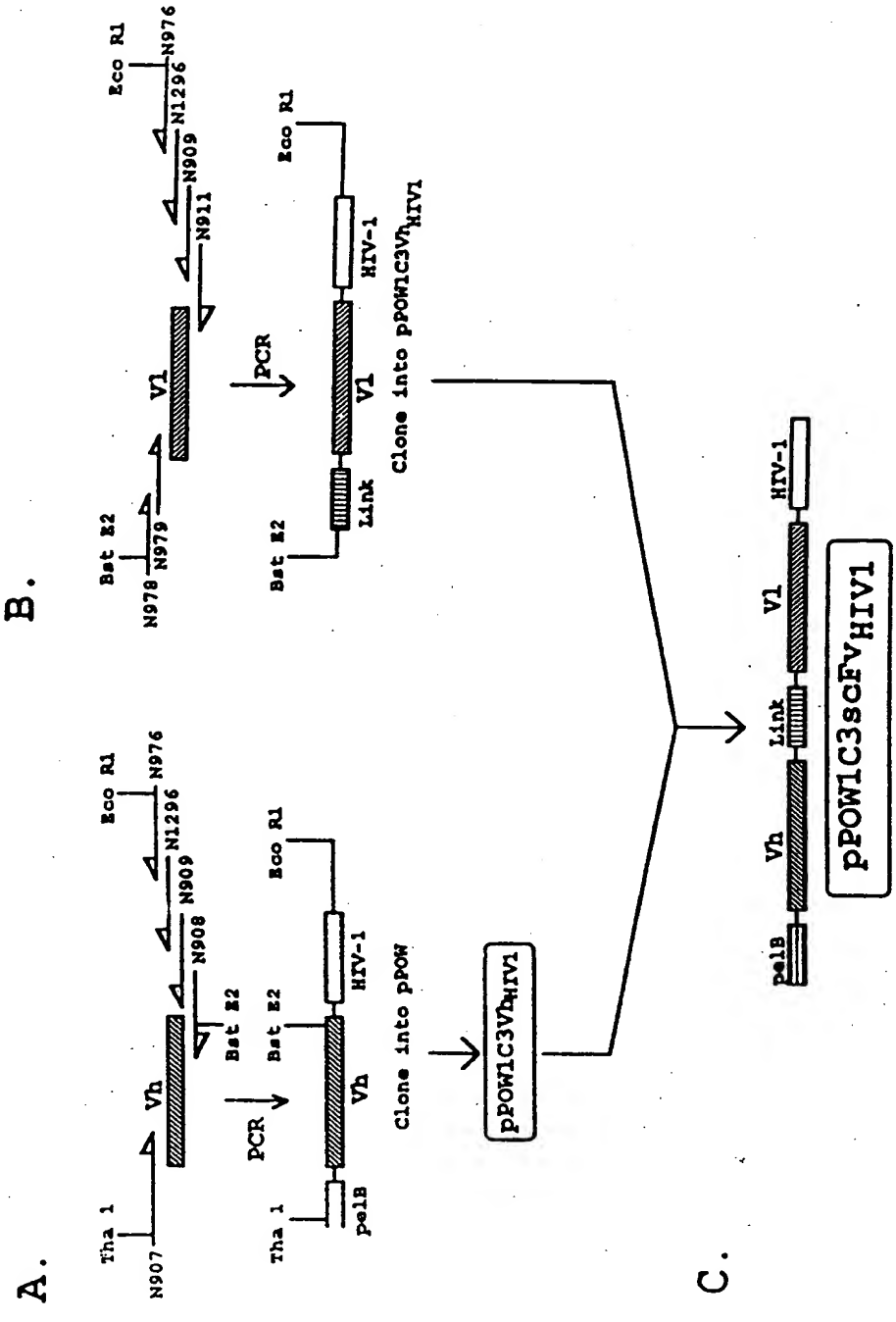

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Figure 4.

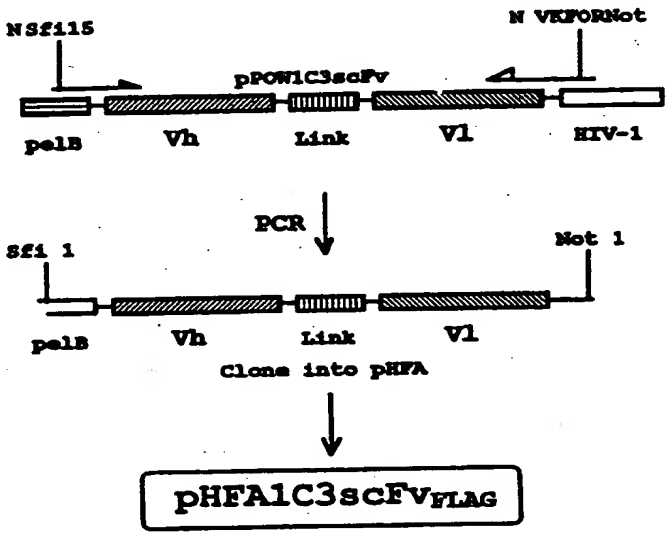
NSf115
N VEFORNot
pPOW1C3scFv
pelB
Vh
Link
Vl
HIV-1
PCR
Sfi 1
Not 1
pelB
Vh
Link
Vl
Clone into pHFA
pHFA1C3scFvFLAG

Figure 5

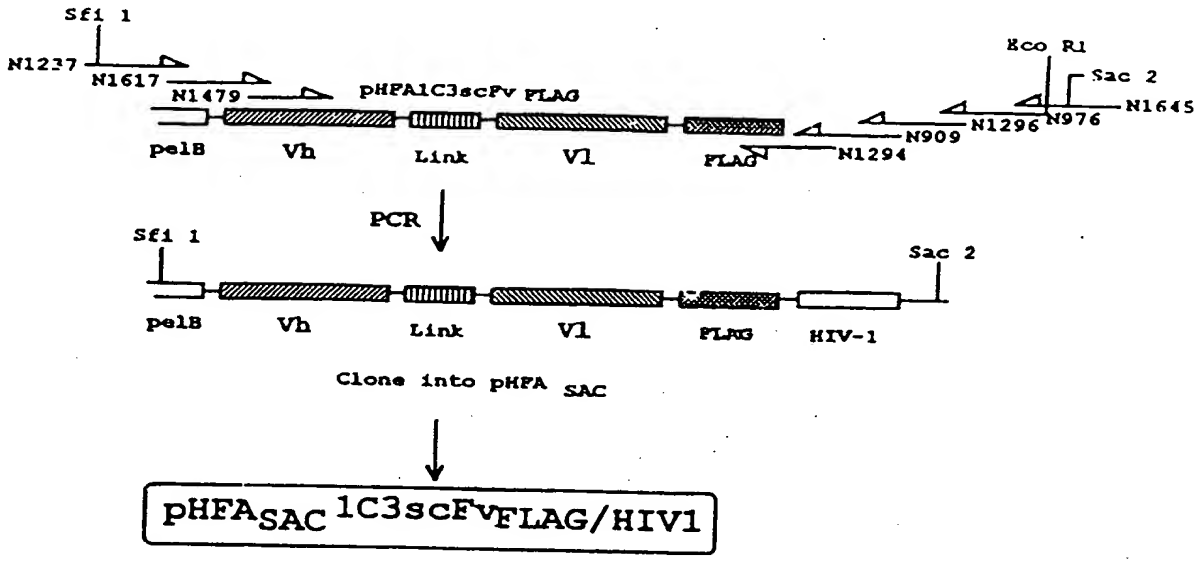

Fig. 5a

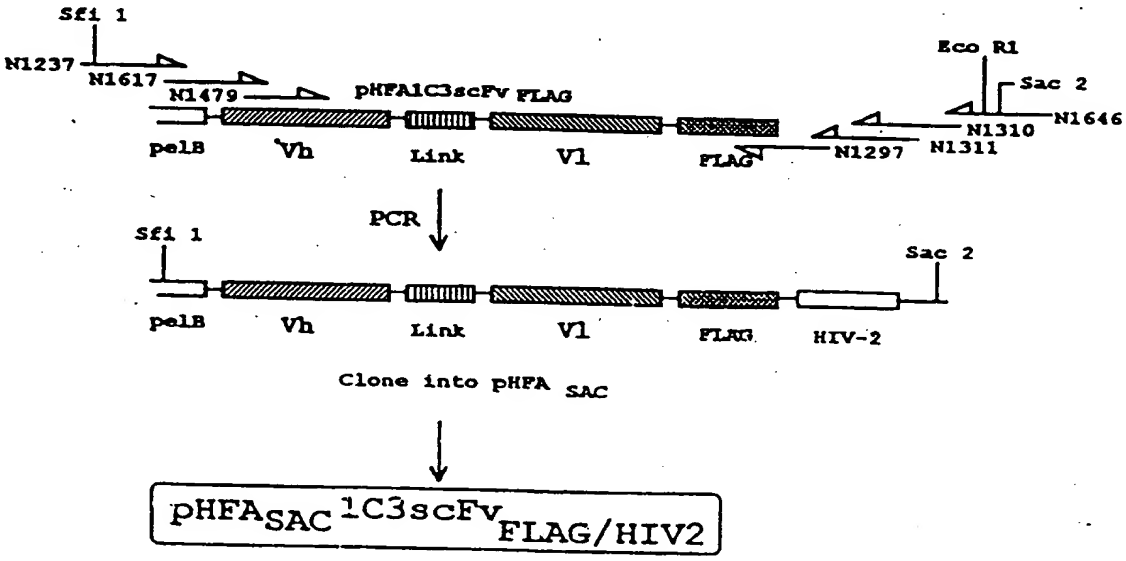

Fig. 5b

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Figure 6

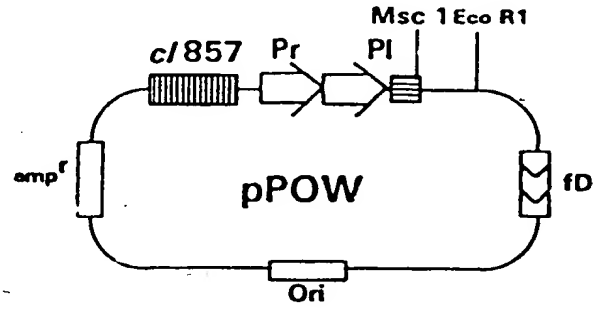

Fig. 6a

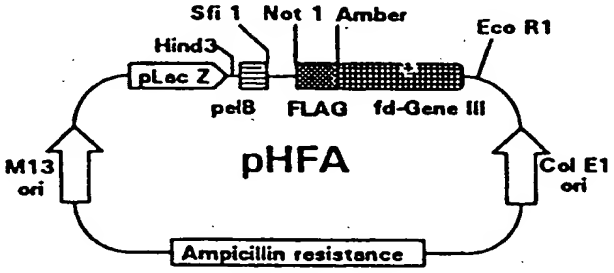

Fig. 6b

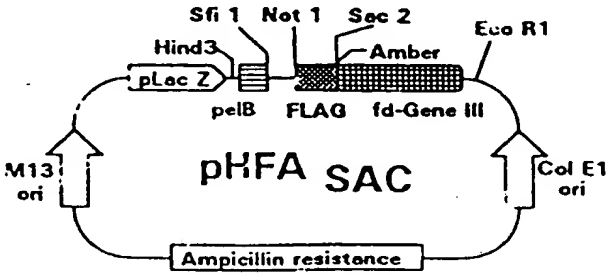

Fig. 6c

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Figure 7.

FLAG™ epitope

NH_2- D Y K D D D D K - COOH

Fig. 7a

gp41 (HIV-1)

NH_2 - R I L A V E R Y L K D Q Q L L G I W-

G C S G K L I C T T A V P W N A S - COOH

Fig. 7b

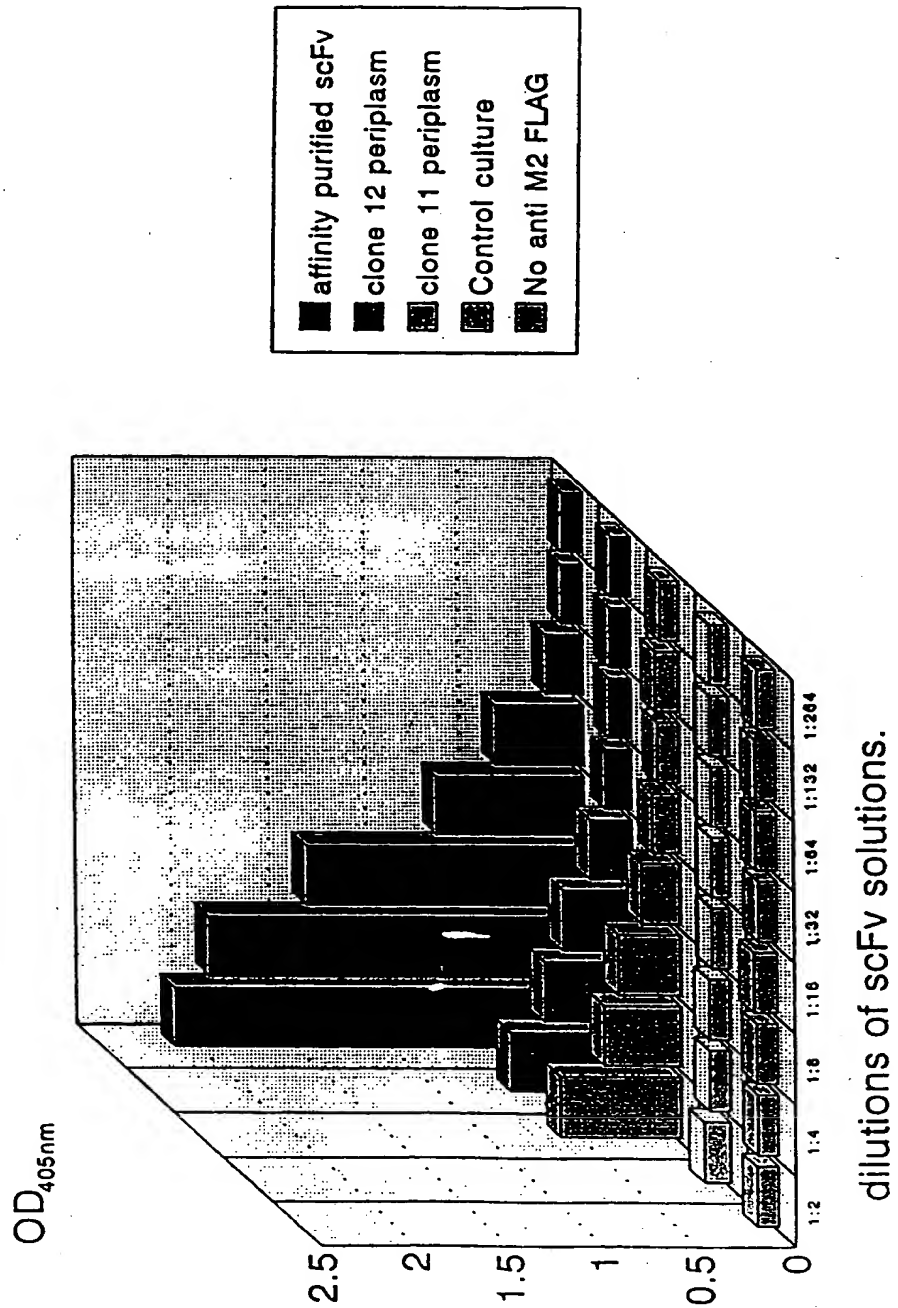
gp35 (HIV-2)

NH_2 - R V T A I E K Y L Q D Q A R L N S W-

G C A F R Q V C - COOH

Fig. 7c

Figure 8


affinity purified scFv
clone 12 periplasm
clone 11 periplasm
Control culture
No anti M2 FLAG
OD405nm
2.5
2
1.5
1
0.5
0
1:2
1:4
1:8
1:16
1:32
1:64
1:132
1:264
dilutions of scFv solutions.

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.[5] C12N 15/13, 15/70 C07K 15/12, 15/28, G01N 33/563, 33/577

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: Derwent Databases: WPAT, CHEM ABSTRACTS, BIOT

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

IPC: AU C12N 15/13

Electronic data base consulted during the international search (name of data base, and where practicable, search terms used)

Derwent Keywords: WPAT - RECOMBIN: BIFUNCTION: BIOSPECIFI: ANTIBOD: ERYTHROGYTE#, RED BLOOD CELL, GLYCOPHORIN# ANTBOD: C12N CHEM ABS + BIOT - AS ABOUT EXCLUDING C12N

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X Y	Journal of cellular biochemistry (suppl) volume 15E (1991) A J T George et al. 'Production of a bispecific antibody by linkage of two recombinant single chain Fv molecules', page 127 (N206)	1, 8-10, 12, 18, 19 4, 22-23
X Y	Journal of Immunological Methods, volume 138 (1991) K M Wilson et al. 'Rapid whole blood assay for HIV-1 seropositivity using an Fab-peptide conjugate', pages 111-119	1, 2, 3, 5, 6, 14, 16, 17, 20-22 4, 22-23

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 July 1993 (09.07.93)	22 July 1993 (22.07.93)

Name and mailing address of the ISA/AU	Authorized officer
AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No. 06 2853929	CARMELA MONGER Telephone No. (06) 2832486

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
X Y	British Medical Journal, volume 305, 6865, (1992), R E Hawkins et al.: 'Adapting antibodies for clinical use' pages 1348-1352, (see especially page 1349 paragraph 2)	1, 8-10, 12, 18, 19 4, 22-23
X	AU 15231/92 (Pasteur Sanofi Diagnostics) 5 November 1992 (05.11.92) (see whole document)	1, 8, 9, 12, 14, 17
X	EP 068763 (The Board of Regents University of Texas System) 5 January 1983 (05.01.83) (see whole document)	1
X Y	AU 59622/90 (Meradex Inc) 10 January 1991 (10.01.91), see especially page 9 lines 7-22	1, 3, 8, 10 4, 22, 23
X	Gene, volume 87 (1990) H Lenz & U H Weidle: 'Expression of heterobispecific antibodies by genes transfected into producer hybridoma cells', pages 213-218	1
Y	Journal of biotechnology, volume 16 (1990) E Kobatake et al.: 'Hyperproduction of a bifunctional hybrid protein metapyrocatechase-protein A, by gene fusion'	1, 8, 12, 13, 14, 15, 19
X	Hybridoma, volume 8 no. 1 (1989), H Tada et al.: 'Bispecific antibody-producing hybrid hybridoma and its use in one-step immunoassays for human lymphotoxin'	1, 14, 15
X	Annals New York Academy of Sciences, volume 646, (1991), J Kohl et al.: 'Cloning and expression of an HIV-1 specific single-chain F v region fused to E coli alkaline phosphatase' pages 106-114	1, 8-12, 18, 19
X	Biotech Forum Eur volume 19, nos. 11-12 (1992) L D Bonino et al.: 'Bispecific monoclonal antibodies - production and clinical application of bispecific antibody by monoclonal antibody engineering; a review pages 722-23 (DBA Accession no. 93-00815)	1
X	Gene volume 122 (1992) A R Gardecha et al.: 'Production and secretion of a bifunctional staphylococcal protein A: antiphytochrome single-chain F v fusion protein in E coli'	1, 8-13, 18, 19
X	WO 91/04492 (Agen Ltd) 4 April 1991 (04.04.91)	1, 2, 5, 6, 14-17
X	US 4894347 (C J Hillyard et al.) 16 January, 1990 (16.01.90)	1-6, 14-17
X	US 5086002 (C J Hillyard et al.) 4 February, 1992 (04.02.92)	1-6, 14-17

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member			
AU	59622/90	EP	479909	WO	9100360
WO	91044/92	AU	64450/90	EP	494210
US	4894347	AU	24182/88		
US	5086002	AU	24182/88		

END OF ANNEX